AD_____

GRANT NUMBER DAMD17-97-1-7291

TITLE: Notch4 Ligands in Mammary Gland Development and Tumorigenesis

PRINCIPAL INVESTIGATOR: Guangyu Wu

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE
Notch4 Ligands in Mammary Gland Development and Tumorigenesis

5. FUNDING NUMBERS
DAMD17-97-1-7291

6. AUTHOR(S)
Guangyu Wu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

To identify and characterize Notch4 ligand, we have obtained several candidate genes and are in the process of testing these genes for their ability to interact with and activate Notch4 receptor. We are trying to determine the locations where Notch4 and its candidate ligands are expressed by immunohistochemistry approach. A luciferase assay based system has been developed to allow fast and quantitative identification of Notch4 ligands. Our preliminary data also indicate that the activation of a Notch receptor can regulate the expression level of itself and other Notch receptors and ligands.

14. SUBJECT TERMS
Breast Cancer
Mammary oncogene, Notch, ligand, tumorigenesis

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

8/31/98

PI - Signature Date

Notch4 Ligands in Mammary Gland Development and Tumorigenesis

Annual Report 8/30/1998

Table of Contents

Introduction

I. Nature of the Problem

Notch/lin-12 receptors have been shown in many cases to regulate cell fate decisions, and the abnormal activation of some of these receptors are also linked to tumorigenesis. Among all the Notch/lin-12 family members, mouse Notch4/int-3 is the only one that has been reported to be expressed in mammary epithelium, and to regulate mammary gland development and tumorigenesis.

A few ligands of the Notch/lin-12 receptors have been identified and as expected, have been shown to regulate cell fate decisions. However, none of these ligands have been investigated for their role in tumorigenesis or mammary gland development. As for Notch4, the only Notch/lin-12 receptor which has been associated with mammary gland development and tumorigenesis, no ligand has been identified yet.

Our proposed study intend to identify Notch4 ligands, study the nature of their interactions with Notch4 receptor and determine their roles in mammary gland development and tumorigenesis. The results of our research will shed light on Notch signaling and mammary gland biology from a new angle.

II. Background

Notch4/int-3 proto-oncogene has been cloned by our lab. *Notch4* gene encodes a 220KD protein product which bears all the hallmarks of a *Notch/lin-12* gene family

member. This protein product is expressed in mouse embryos and a number of adult tissues including lung, kidney, heart and mammary gland(1, 2). *In situ* hybridization data showed that Notch4 expression in day 13.5 embryo and adult lung is endothelial cell specific(1). Our unpublished immunofluorescent staining data have confirmed that Notch4 is a cell surface localized protein.

Truncated Notch proteins have been shown to be involved in tumorigenesis. In *Drosophila* and other organisms, truncated Notch proteins containing the intracellular domain behave like constitutively activated receptors(3). The truncated form of *TAN-1* gene was first isolated from patients with acute T lymphoblastic leukemia(4), and was later shown to promote T cell neoplasm in bone marrow reconstitution assay(5) and to transform rat kidney cells in soft-agar assay(6). The truncated *Notch4* gene, *int-3* was identified as a target of intergration by mouse mammary tumor virus (MMTV) in mouse mammary tumors. Viral integration into the *int-3* gene results in the expression of a truncated 2.4kb transcript(7). Transgenic mice that express *int-3* under the control of either MMTV LTR or whey acidic protein (WAP) promoter develop poorly differentiated mammary adenocarcinomas at 100% penetrance(8, 9). Histological examination showed that the mammary glands of the transgenic mice were arrested during development and were lactation deficient(8). int-3 has also been demonstrated to be oncogenic in cultured mammary epithelial cells(7).

Although Notch receptors activated by truncation have been extensively studied for their role in development and tumorigenesis, Notch receptors activated by ligand have never been analyzed in the same scenario. Genetic and molecular analyses have identified several Notch ligands in *Drosophila* and *C. elegans*. *Drosophila Delta* and *Serrate* genes and *C. elegans Lag-2* and *Apx-1* genes encode a family of structurally related ligands for the *Drosophila* Notch and the *C. elegans* lin-12 and glp-1 receptors(10, 11). These ligands

are all membrane bound proteins of which the extracellular domains contain a variable number of EGF-like repeats and a cysteine-rich DSL (Delta-Serrate-Lag-2) motif. Although in general these structural motifs are believed to participate in ligand-receptor interaction, it is not very clear what specific regions are involved in receptor binding and how they interact with the receptor. The function of the very short intracellular domain of Notch ligands is not clear either. Notch ligands have also been identified in mammals. *Jagged-1*, a rat homologue of *Serrate*, was cloned from Schwann cell cDNA library by low stringency hybridization(12). It has all the typical structures of Notch ligands. Jagged-1 is co-expressed with Notch1 in developing spinal cord and other tissues. Jagged-1 activated Notch1 blocks in vitro myogenesis in a way very similar to the truncated receptor. Other mammalian Notch ligands, such as human Jagged-1 and Jagged-2, mouse Delta-like 1 (Dll1) and Dll3 have also been cloned and shown to be involved in different developmental processes. For example, human Jagged-1 mutations have been found to be responsible for Alagille syndrome, a genetic disorder characterized by abnormal development of liver, heart, skeleton, eye, face and kidney. However, the role of these mammalian Notch receptors in tumorigenesis has never been determined. *C. elegans* Apx-1, although has different receptor and different mutant phenotype from Lag-2, can fully substitute Lag-2 when expressed under the control of *lag-2* regulatory sequences(13). Similarly, *Drosophila* Serrate can functionally replace Delta during neuroblast segregation in the *Drosophila* embryo(14). This implicates that different Notch receptors and ligands function through a similar mechanism.

Genetic and molecular studies have also identified intracellular components of Notch signaling pathway. *Drosophila* gene *Suppresser of Hairless (Su(H))* may play a central role in Notch signaling. Su(H) protein is sequestered in the cytoplasm when co-expressed with Notch protein in cultured *Drosophila* S2 cells and is translocated to the nucleus when Notch binds to its ligand Delta(15). In mammalian cells, truncated mouse

Notch1 and Notch2 have been shown to be localized in the nucleus and interacts directly with RBP-Jκ (recombination signal sequence binding protein for Jκ genes), a transcriptional factor highly related to Su(H). The binding of truncated Notch1 and Notch2 to RBP-Jκ activates the expression of RBP-Jκ repressed genes, such as Hairy Enhancer of Split (HES-1)(16, 17, 18) The fact that truncated Notch proteins without the extracellular domains are always localized to the nucleus has led to the speculation that a cleaved fragment of wild-type Notch receptor may participates directly in the downstream nuclear events of Notch signaling. Recent studies have provided very strong evidence to support this model(19, 20).

III. Purpose

The overall goal of my proposed work is to find Notch4 ligands and determine their role in mammary gland development and tumorigenesis.

Body

I. Technical Objectives

The goals of this research project is to identify ligands for mouse Notch4 receptor, understand in molecular details of their interaction with Notch4, and determine their involvement in mammary gland development and tumorigenesis.

I proposed two specific aims to achieve the above goals in a period of three years:

Specific Aim 1: Identifying and Cloning Genes that Encode Putative Notch4 Ligands (MONTHS 1-18)

In mouse, several genes have been identified as Notch ligands, such as *Jagged* and *Delta-like* genes. Since some Notch ligands are interchangeable, we will test whether the protein products of these genes are able to interact with and activate Notch4 receptor.

In addition, we will try to identify novel putative ligands for Notch4 by a screening strategy. Extracellular domain of Notch4 receptor will be used as a molecular probe to screen an eukaryotic expression library from mouse mammary gland. Once a novel gene is identified, we will obtain its full length cDNA for further investigation.

Specific Aim 2: Biochemical and Biological Interactions between Putative Ligands and Notch4 Receptor (MONTHS 18-36)

To demonstrate that a candidate protein is the bona fide Notch4 ligand, we will first show its physical interaction with Notch4 receptor by molecular and cellular techniques. As a property of EGF repeat contain proteins, their interaction should be Ca^{++} dependent. We then plan to analyze the expression patterns of the candidate ligands and Notch4 receptor. This experiment will not only help us to rule out ligands for other Notch receptors that can cross-interact with Notch4 but also inform us whether Notch4 has different ligands in different tissues. Furthermore, by functional assays, we will test the candidate ligands for their ability to activate Notch4 signaling pathway. Finally, we will also test Notch4 ligands for their ability to regulate mammary epithelial cell development by using transformation and differentiation assays.

II. Experimental Results

This annual report describes the progress we have made during the first 12 months of this fellowship. The progress reported here will be related to the original tasks set out in the Statement of Work.

Specific Aim 1: Identifying and Cloning Genes that Encode Putative Notch4 Ligands (MONTHS 1-18)

A. Obtaining full length cDNA clones of mouse Jagged and Delta-like 1. We have obtained rat Jagged-1 cDNA, epitope-tagged it and cloned the tagged gene into retroviral vector and adenovirus vector. At the same time, we also cloned Notch4 and int-3 genes into retroviral and adenovirus vectors. These vectors have been successfully used to drive stable or transient gene expression in different cells. A Western blot showing the expression levels of the three proteins is attached in Appendix A.

We have also acquired mouse Developmental Endothelial Locus-1 (Del1) gene which encodes a EGF repeat containing protein in endothelial cells, the same location where Notch4 is expressed. We will test Del1 as a candidate Notch4 receptor. We are also in the process of acquiring more candidate genes, such as mouse Delta-like 1 (Dll1) and Dll3, Jagged-2, and so on. All these candidate Notch4 ligands will be tested for their ability to interact with Notch4 biochemically and biologically.

B. Screening mammary gland eukaryotic expression libraries for putative Notch4 ligand. For this approach, we planned to determine the locations where Notch4 ligands are most concentrated and then screen an expression library made from such locations. Northern blot and in situ hybridization have shown that Notch4 is expressed in endothelial cells. To confirm this result, we have been trying to study the expression pattern of Notch4 in adult mouse tissues by immunohistochemistry. A well established expression pattern of Notch4 will be a good indication of where its ligands are located.

We used a rabbit polyclonal antiserum against the C-terminal region of Notch4 to probe for Notch4 expression in adult mouse kidney tissue sections. The characteristic glomeruli with endothelial cell clusters in the renal cortex are very easy to identify, and that makes kidney a perfect organ to establish the conditions of immunohistochemical analysis using our antiserum. Together with anti-Notch4 antibody, we also used pre-immune serum in our immunohistochemical staining as negative control and anti-PECAM (an endothelial marker) antibody as positive control. Our preliminary results strongly indicate that Notch4 is expressed in kidney glomeruli. However, high background has prevented us from producing publication quality photos. To solve this problem, we are now trying to purify our anti-Notch4 antiserum using affinity chromatography.

The Notch1 ligand, Jagged-1 has been reported to be expressed in endothelial cells. It is reasonable to ask whether Jagged-1 can also serve as a Notch4 ligand. In our immunohistochemistry studies using kidney sections, we also include an affinity purified Jagged-1 antibody. The staining pattern of Jagged-1 is clearly endothelial and is very similar to Notch4 expression pattern.

Specific Aim 2: Biochemical and Biological Interactions between Putative Ligands and Notch4 Receptor (MONTHS 18-36)

A. Co-immunoprecipitation of Notch4 and its putative ligands. We are in the process of testing Notch4 and Jagged-1 in co-immunoprecipitation experiments. Jagged-1 has been HA-tagged and will be co-expressed with Notch4 by transient transfection into Bosc23 cells. Anti-Notch4 and anti-HA antibodies will be used to precipitate and detect the protein products.

B. Cell aggregation assay to show physical interactions between Notch4 receptor and ligands. This experiment has not been carried out yet. But we have gathered all the necessary reagents, and once a promising candidate gene is available, we will test it in this assay.

C. Transformation assays to show activation of Notch4 receptor by its ligands.

D. Differentiation assays to show activation of Notch4 receptor by its ligands.

We have not carried out experiments proposed in C and D. Instead, we have developed a luciferase assay to test Notch4 candidate ligands in a faster and more quantitative way. Once a Notch4 ligand is identified in the luciferase assay, we eventually

will test its role in mammary gland development and tumorigenesis by transformation and differentiation assays using mammary epithelial cells.

E. Immunofluorescent staining to study the subcellular localization of Notch4 receptor before and after its activation by ligand. Recently studies have provide quite convincing evidence supporting the model that Notch activation by its ligands induces a proteolytic processing resulting in the translocation of the intracellular domain of the receptor to the nucleus. Only a small amount of the truncated protein is required to exert its nuclear function. That is why it is very hard to detect the nuclear fragment caused by ligand binding. We still plan to do the proposed immunofluorescent staining experiment at a convenient time. But it will not be a top priority in the overall plan.

F. Luciferase assays to show activation of Notch4 receptor by its ligands. This experiment was not proposed in my original proposal. The intracellular domain of Notch1 has been shown to be able to activate a luciferase reporter gene under the control of a HES-1 promoter(21). We replaced Notch1 intracellular domain with int-3 or Notch4 and showed that int-3 can activate HES-1 transcription while Notch4 can not. We are going to co-express Jagged-1 and Notch4 in HELA cells using adenovirus and determine if Jagged-1 can activate Notch4 and lead to luciferase expression. This experiment is much faster and easier to carry out then transformation and differentiation assays. It will be our major approach to identify Notch4 ligand.

G. Cross-regulation among Notch receptors and their ligands. This experiment was not proposed in my original proposal. In our experiments designed to study the function of Notch receptors in endothelial cells, we found that the expression of one Notch receptor or ligand can sometimes up-regulate or down-regulate the expression levels of other Notch receptors or ligands. For example, we have found by Northern blot that

Notch1, Notch3, Notch4 and Jagged-1 are all expressed in RBE4 cells, a rat brain endothelial cell line. Exogenous expression of the activated form of Notch4, int-3 increases the steady state levels of both endogenous Notch4 and endogenous Jagged-1 (Appendix B). We will assess this cross-regulation in further details and try to determine the specificity between Notch ligand and receptor pairs. This study has the potential to develop into a new functional analysis system for Notch ligand-receptor interaction.

Conclusions

The data presented in this annual report represent our progress in the experiments outlined in the specific aims of the research proposal. In the past year, we have obtained several candidate Notch4 ligand genes and have developed a series of reagents that will enable us to carry out biochemical and biological studies. We have also developed functional analysis systems in which we can quickly and easily test a candidate protein for its ability to bind and activate Notch4 receptor. Our observation that cross-regulations exist among different Notch pathway genes brings up an interesting angle to understand Notch ligand-receptor interaction and the potential to develop a new assay for ligand dependent Notch activation.

References

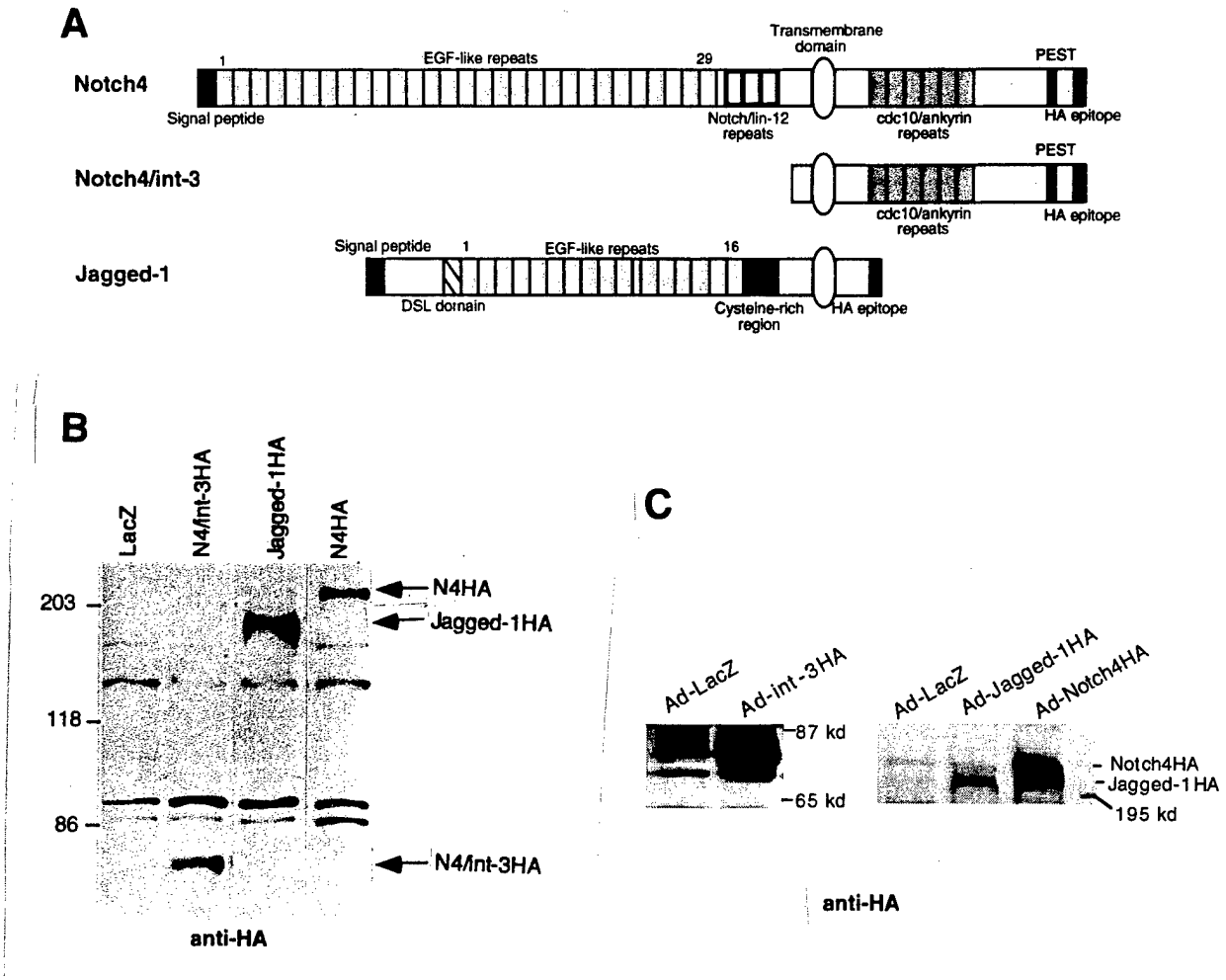
1. H. Uyttendaele, et al., *Development* **122,** 2251-2259 (1996).
2. G. H. Smith, et al., *Cell Growth & Diff.* **6**, 563-77 (1995).
3. G. Struhl, K. Fitzgerald, I. Greenwald, *Cell* **74**, 331-345 (1993).
4. L. W. Ellisen, et al., *Cell* **66**, 649-661 (1991).
5. W. S. Pear, et al., *J. of Exp. Med.* **183**, 2283-2291 (1996).
6. A. J. Capobianco, P. Zagouras, C. M. Blaumueller, S. Artavanis-Tsakonas, J. M. Bishop, *Mol Cell Biol* **17**, 6265-73 (1997).
7. J. Robbins, B. J. Blondel, D. Gallahan, R. Callahan, *J. of Virology* **66**, 2594-2599 (1992).
8. C. Jhappan, et al., *Genes & Dev.* **6**, 345-355 (1992).
9. D. Gallahan, et al., *Cancer Res.* **56**, 1775-85 (1996).
10. S. Artavanis-Tsakonas, K. Matsuno, M. E. Fortini, *Science* **268**, 225-232 (1995).
11. J. S. Nye, R. Kopan, *Curr. Biol.* **5**, 966-969 (1995).
12. C. E. Lindsell, C. J. Shawber, J. Boulter, G. Weinmaster, *Cell* **80**, 909-917 (1995).
13. K. Fitzgerald, I. Greenwald, *Development* **121**, 4275-4282 (1995).
14. Y. Gu, N. A. Hukriede, R. J. Fleming, *Development* **121**, 855-865 (1995).
15. M. E. Fortini, S. Artavanis-Tsakonas, *Cell* **79**, 273-282 (1994).
16. J. J. Hsieh, et al., *Mol Cell Biol* **16**, 952-959 (1996).
17. J. J. Hsieh, D. E. Nofziger, G. Weinmaster, S. D. Hayward, *J Virol* **71**, 1938-45 (1997).
18. F. M. Lu, S. E. Lux, *Proc. Natl. Acad. Sci. USA* **93**, 5663-5667 (1996).
19. G. Struhl, A. Adachi, *Cell* **93**, 649-660 (1998).
20. E. H. Schroeter, J. A. Kisslinger, R. Kopan, *Nature* **393**, 382-386 (1998).
21. S. Jarriault, et al, *Nature* **377**, 355-8 (1995).

Appendices

Appendix A Western blots of int-3, Notch4 and Jagged-1 proteins

Appendix B Northern blot analysis on RBE4 cells

Appendix A

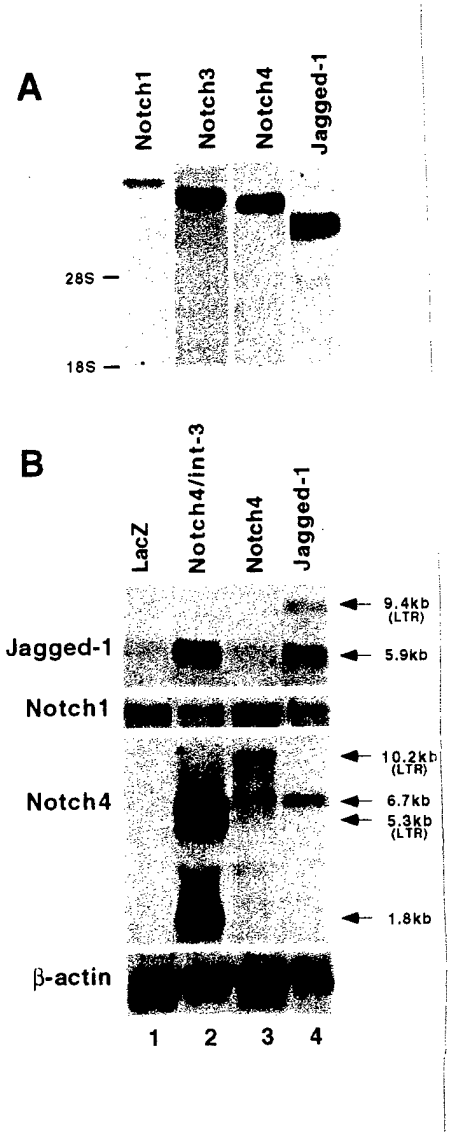


A. Schematic representation of the HA-tagged Notch4, int-3 and Jagged-1 proteins.

B. RBE4 cells infected by retrovirus stably express int-3, Notch4, and Jagged-1 proteins.

C. HELA cells infected by adenovirus (Multiplicity of Infection=10) carrying *LacZ, int-3HA, Notch4HA* and *Jagged-1HA* genes.

Appendix B



A. Notch1, Notch3, Notch4 and Jagged are all expressed in RBE4 cells. 40ug of total RNA from RBE4 cells hybridized to riboprobes for either *Notch1, Notch3, Notch4* or *Jagged-1.*

B. Cross-regulation of Notch receptors and Ligand. 40ug of total RNA (lane 1, 3) or 20ug of total RNA (lane 2, 4) from RBE4 cells expressing LacZ, int-3, Notch4 or Jagged-1 from a CMV promoter, was hybridized to riboprobes for *Jagged-1, Notch1, Notch4* or *β-actin.* int-3 expression up-regulates the expression of endogenous Notch4 and Jagged-1. RBE4 cells stably expressing Notch receptor or ligand were generated by retroviral infection. RNA labeled with "LTR" indicates retroviral genome RNA that is transcribed from the LTR promoter.